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Review

X-Inactivation, Imprinting, and Long Noncoding RNAs in Health and Disease

Jeannie T. Lee[1,2,*] and Marisa S. Bartolomei[3,*]
[1]Howard Hughes Medical Institute, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA
[2]Department of Genetics, Harvard Medical School, Boston, MA 02114, USA
[3]Department of Cell and Developmental Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA
*Correspondence: lee@molbio.mgh.harvard.edu (J.T.L.), bartolom@mail.med.upenn.edu (M.S.B.)
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X chromosome inactivation and genomic imprinting are classic epigenetic processes that cause disease when not appropriately regulated in mammals. Whereas X chromosome inactivation evolved to solve the problem of gene dosage, the purpose of genomic imprinting remains controversial. Nevertheless, the two phenomena are united by allelic control of large gene clusters, such that only one copy of a gene is expressed in every cell. Allelic regulation poses significant challenges because it requires coordinated long-range control in *cis* and stable propagation over time. Long noncoding RNAs have emerged as a common theme, and their contributions to diseases of imprinting and the X chromosome have become apparent. Here, we review recent advances in basic biology, the connections to disease, and preview potential therapeutic strategies for future development.

Introduction

Every organism faces the challenge of regulating gene dosage. In diploids, genes are generally assumed to be expressed from both alleles but, in mammals, several classes of genes are expressed from only one allele per cell. Two of the most prominent examples of allelic phenomena are X chromosome inactivation (XCI) and genomic imprinting. Because of XCI, only one copy of each X-linked gene is active in female cells (XX). Because male cells carry only one X chromosome (XY), they are inherently hemizygous for all X-linked genes. In genomic imprinting, genes within a discrete domain are coordinately regulated and expressed according to parent of origin. Research over the past 50 years has identified many similarities between XCI and genomic imprinting. Apart from monoallelic expression, genes subject to the two processes tend to cluster, are influenced at long-range by a master control region, and are associated with multiple long noncoding RNAs (lncRNA). Some of the most fascinating stories to emerge in recent years have been related to lncRNAs as master regulators. Some of the first epigenetic lncRNAs in mammals were, in fact, identified from genomic imprinting and XCI studies. Such lncRNAs have been proposed to serve as recruiting tools for chromatin-modifying complexes that would in turn silence or activate genes residing within the allelically regulated clusters.

Together, XCI and imprinting affect expression of ~5%–10% of genes in the mammalian genome. From a functional standpoint, mutations within these regions could be easily unmasked, as they are often unbuffered by contributions from the silenced wild-type copy and could thereby cause severe developmental defects. This explains why X-linked and imprinted diseases are among the most common congenital human disorders, accounting for easily recognizable childhood syndromes such as Rett, fragile X, Prader-Willi/Angelman, and Beckwith-Wiedemann syndromes, as well as conditions such as hemophilia, testicular feminization, and red-green color blindness. More recently, imprinting and X-linked anomalies have also been of interest for stem cell maturation and reprogramming, cancer, assisted reproductive technology (ART), and cognition. This article will review the state-of-the-art in genomic imprinting and XCI, focusing on recent advances in studying mechanism, the emerging roles of lncRNAs, and their relevance for understanding and treating various human conditions.

X Chromosome Inactivation and Genomic Imprinting

Genomic Imprinting

Mammals require both maternal and paternal genomic contributions to develop into healthy, viable organisms (Solter, 1988). This is, in large part, due to the inheritance of autosomally imprinted genes, which are expressed only from a single allele in accordance with its parent of origin (Bartolomei, 2009). That is, imprinted genes are expressed either from the maternally or paternally inherited allele, so that, when summed across the whole genome, contributions from both parents are necessary for expression of the full complement of imprinted genes and for proper development. The elegant nuclear transplantation experiments by Solter and Surani in the 1980s were the first to suggest that the mammalian genome harbored imprinted genes (McGrath and Solter, 1984; Surani et al., 1984). They showed that diploid androgenetic embryos derived from two male pronuclei or diploid gynogenetic embryos derived from two female pronuclei failed to develop and reasoned that this must be due to genes that are expressed exclusively from one of the parental genomes. Later genetic experiments extended these findings by demonstrating that the proposed imprinted

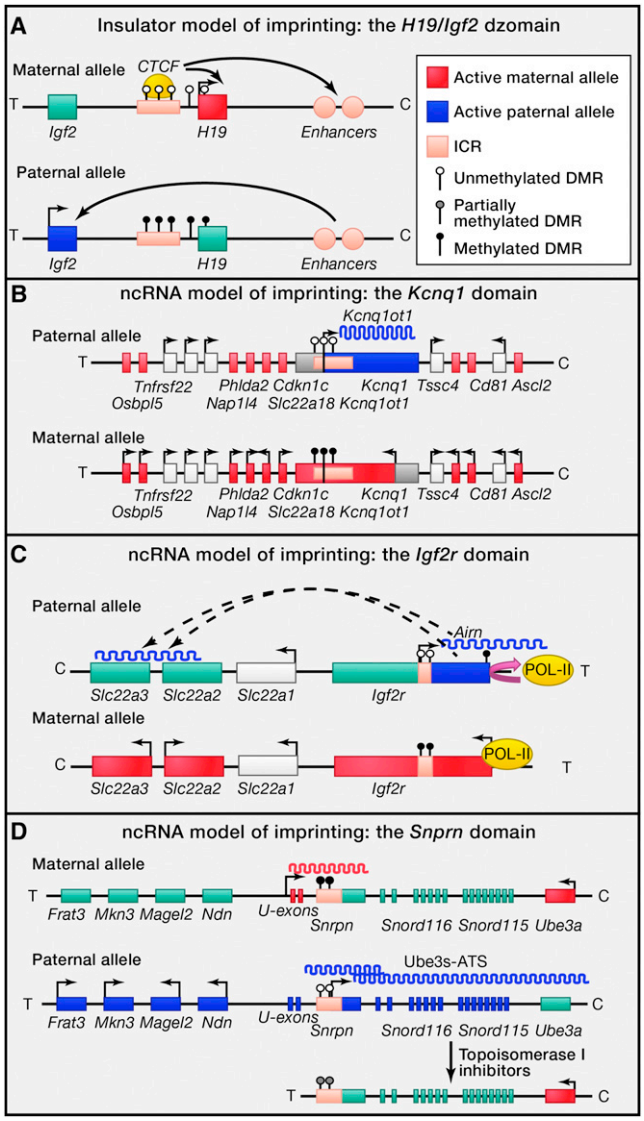

Figure 1. Mechanisms of Imprinting

(A)The insulator model is exemplified by the *H19/Igf2* domain. Here, the intergenic ICR is paternally methylated. On the unmethylated maternal allele, CTCF binding prevents enhancers from interacting with the *Igf2* promoter. Instead, the enhancers activate *H19* expression. On the paternal allele, methylation of the ICR spreads to the *H19* promoter, silencing its expression, and prevents CTCF from binding the ICR, allowing the enhancers to activate *Igf2* expression.

(B–D) The ncRNA model is illustrated by the *Kcnq1* (B), *Igf2r* (C), and Snprn (D) domains.

(B) For *Kcnq1*, the ICR contains the promoter of the *Kcnq1ot1* lncRNA. On the paternal allele, the ICR is unmethylated, allowing *Kcnq1ot1* expression. *Kcnq1ot1* expression silences the paternal allele of the linked genes in *cis*. On the maternal allele, *Kcnq1ot1* is not expressed due to methylation of the ICR, and the adjacent imprinted genes are expressed.

(C) For the *Igf2r* domain, transcription of the Airn lncRNA is governed by a promoter within the ICR and is expressed from the unmethylated paternal allele. In somatic cells, transcription of Airn over the *Igf2r* promoter precludes *Igf2r* expression, in part by kicking RNA polymerase II (POL-II) off of the promoter. In extraembryonic lineages, Airn lncRNA is postulated to recruit enzymes that confer repressive histone modifications to silence genes in *cis*.

(D) The *Snrpn* locus uses the ncRNA model. *Ube3a* is expressed from the maternal allele exclusively in brain (in other tissues, it is biallelically expressed). The lncRNA on the paternal allele occurs in multiple, variably processed forms, some of which are brain-specific variants that contain upstream promoters/exons and sequences overlapping with *Ube3a*. Expression of these lncRNAs occurs when the ICR is unmethylated, with the result that expression of *Ube3a* is repressed. On the maternal allele, transcription of the upstream (U) exons is proposed to direct the maternal methylation imprint at the ICR. Topoisomerase I inhibitors identified by a mouse screen activate *Ube3a* on the paternal allele. As a result, *Snprn* and *Ube3a-ATS* were no longer expressed and the ICR exhibited increased methylation, relative to the wild-type paternal allele.

All imprinted domains, which are not drawn to scale, are depicted for the mouse, although the human regions are largely conserved. T refers to the telomeric end of the cluster and C the proximal end of the chromosome.

genes mapped to specific mouse chromosomes (Searle and Beechey, 1978; Cattanach, 1986).

The current number of imprinted genes in the mouse is approximately 150 (http://www.mousebook.org/catalog.php?catalog=imprinting), with a smaller number identified in humans, in part because many genes have not been tested in humans (Weksberg, 2010). The imprinted genes are typically located in clusters of 3–12 (or more) genes that are spread over 20–3,700 kb of DNA (Barlow, 2011), but, interestingly, genes within one cluster are not necessarily expressed from the same parental chromosome (Figure 1). Most imprinted clusters contain protein coding genes and noncoding RNAs (ncRNAs). The ncRNAs are of different varieties (microRNAs, snoRNAs, and lncRNAs), some of which are essential to the mechanism that imprints these genes in *cis*. Each well-studied cluster has a discrete imprinting control region (ICR) that exhibits parent-of-origin-specific epigenetic modifications (DNA methylation and posttranslational histone modifications) and governs the imprinting of the locus. Although the mechanism(s) that confer the allele-specific epigenetic modifications is poorly understood, DNA methylation has been shown to be imposed at a precise time in germ cells by a mechanism that is postulated to involve transcription (Chotalia et al., 2009; Henckel et al., 2012) and is maintained after fertilization despite extensive reprogramming of the genome (Bartolomei and Ferguson-Smith, 2011) (Figure 1). Moreover, germline deletion of the ICR results in the loss of imprinting of multiple genes in the cluster, thus demonstrating that the clustering of imprinted genes is required for their appropriate expression.

Many imprinted genes undergo tissue-specific imprinting. Of the approximately 150 imprinted genes identified in mouse, a few are imprinted exclusively in the placenta, an extraembryonic organ that plays a crucial role in regulating fetal growth by controlling the supply of nutrients (Frost and Moore, 2010). Imprinting is hypothesized to be a mechanism to balance growth, with many imprinted genes having a demonstrated role in growth control. Thus, the placenta is particularly significant in the physiology and study of imprinting. Interestingly, as described below, mechanisms that control imprinting in the placenta—a short-lived organ—may differ from those mechanisms that regulate imprinting in the much longer-lived somatic lineages. This holds true not only for autosomal but also X-linked imprinting.

X Chromosome Inactivation

In 1949, Murray Barr showed that the sex of cat cells could be deduced by a subnuclear structure now called the "Barr body" in honor of his seminal work (Barr and Bertram, 1949). Susumu

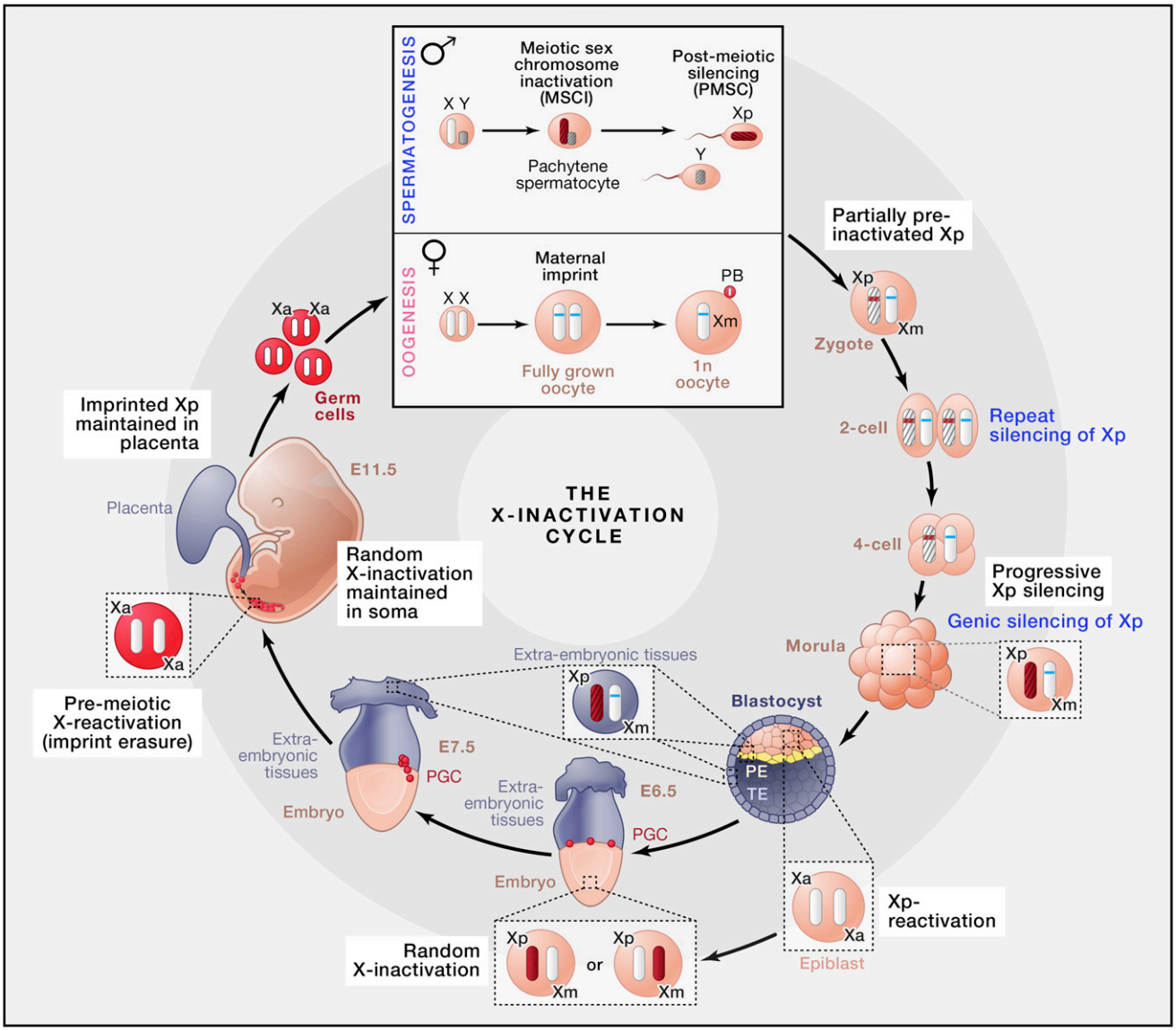

Figure 2. The X-Inactivation and X-Reactivation Cycle during Mouse Development

Mammalian dosage compensation occurs within a continual cycle of XCI and X reactivation. The XCI cycle begins in the male germline. During the first meiotic prophase of spermatogenesis, the X and Y undergo MSCI. After meiosis, 85% of X-linked genes remain suppressed through spermiogenesis, forming postmeiotic sex chromatin (PMSC). This germline-inactivated X has been proposed to be passed onto the next generation in a partially silent state, accounting for the preferential X^P inactivation of the early female mouse embryo. In the two-cell mouse embryo, repetitive elements on X^P are already suppressed. X^P-linked coding genes are initially active but become progressively inactivated during preimplantation development. The maternal germline also plays a crucial role in imprinted XCI by marking the future X^M during the oocyte growth phase, ensuring that X^M is protected in both XX and XY embryos. Beyond the blastocyst, these marks persist only in the placenta of the mouse. Whereas extraembryonic tissues, including the primitive endoderm (PE) and the trophectoderm (TE), maintain imprinted X^P inactivation, the epiblast lineage undergoes XCR and initiates zygotically driven random XCI. XCR also occurs in primordial germ cells (PGCs) in preparation for equal segregation during meiosis. Xp, paternal X; Xm, maternal X; Xa, active X; MSCI, meiotic sex chromosome inactivation. Adapted from Payer and Lee, 2008.

Ohno later demonstrated that the Barr body is a condensed X chromosome (Ohno et al., 1959), and Mary Lyon followed with the understanding that the condensed X is the result of whole-chromosome silencing (Lyon, 1961). We now know that XCI compensates for dosage differences between males and females by rendering all cells functionally monosomic for the X chromosome (reviewed in Payer and Lee, 2008; Starmer and Magnuson, 2009; Wutz, 2011). XCI is coordinated by an X-inactivation center (*Xic*), which controls most, if not all, of the steps of XCI, including X chromosome counting, random X chromosome choice, and the initiation of silencing along ~1,000 genes of the X (Brown et al., 1991b) (Figure 2). These steps are completed in the peri-implantation embryo within the 10–20 cell epiblast lineage (which gives rise to all somatic cells) (Puck et al., 1992).

Once established, the pattern of XCI is stably propagated in the soma, with the same X chromosome maintained as Xi in subsequent mitotic divisions. The mammalian female is therefore a mosaic (Figure 2).

Whereas the choice of XCI in somatic cells of eutherian mammals occurs randomly, the choice in marsupial mammals is fixed. In marsupials (Sharman, 1971), and also in the extraembryonic tissues of some eutherian mammals (Takagi and Sasaki, 1975), the paternal X (X^P) is imprinted to undergo silencing, providing a first example of mammalian imprinting. The phenomenon is conceptually similar to autosomal imprinting in that monoallelic expression is determined by parent-of-origin and has mechanistic underpinnings in the parental germline. Imprinted XCI in the placenta adds to the number of imprinted mammalian genes and further supports the idea that imprinting balances fetal growth by controlling the nutrient supply (Frost and Moore, 2010).

Mammalian dosage compensation occurs within a continual cycle of XCI and reactivation (XCR) (Figure 2). Although random XCI is female-specific, X silencing also occurs in the male germline (Lifschytz and Lindsley, 1972). For some mammals, the male germline is where the XCI cycle begins. During the first meiotic prophase of spermatogenesis, the X and Y undergo "meiotic sex chromosome inactivation" (MSCI) and form the "XY body". The X and Y do not wholly reactivate after completion of meiosis; in mice, 85% of genes on the X chromosome remain transcriptionally suppressed in postmeiotic spermiogenesis (Namekawa et al., 2006). This "postmeiotic sex chromatin" (PMSC) is decorated by distinct heterochromatic signatures (Greaves et al., 2006; Namekawa et al., 2006; Turner et al., 2006) and is consistent with the hypothesis that the germline-inactivated X may be passed onto the next generation at least in a partially preinactivated state, accounting for the preferential X^P inactivation that occurs in the early female embryo (Cooper, 1971; Lyon, 1999; Huynh and Lee, 2003).

At zygotic gene activation in the two-cell mouse embryo, transcription of repetitive elements on X^P is already suppressed, reflecting their suppression in the male germline (Namekawa et al., 2006; Namekawa et al., 2010) (Figure 2). Although X-linked coding genes on the X^P are initially active, they are progressively inactivated during preimplantation development (Okamoto and Heard, 2006; Kalantry et al., 2009; Namekawa et al., 2010). The X-linked repetitive elements may facilitate formation of the silent compartment for inactivation of X^P genes (Namekawa et al., 2010). Thus, imprinted XCI may be a process that begins in the male germline, continues into the zygote as repeat silencing, and progresses through the blastocyst stage with genic silencing. However, the maternal germline also plays a crucial role in imprinted XCI by marking the future X^M to resist silencing (Takagi and Abe, 1990; Goto and Takagi, 2000). This occurs during the oocyte growth phase (Tada et al., 2000), ensuring that X^M (passed onto both XX and XY embryos) is protected (Figure 2). Thus, it is likely that both X^P and X^M are parentally marked, with X^P subject to imprinted XCI and X^M protected from it.

Beyond the blastocyst, these marks persist only in the placenta of the mouse (Figure 2). The blastocyst consists of the trophectodermal lineage, which gives rise to placental tissue, and the inner cell mass, which gives rise to the epiblast lineage that develops into the embryo proper. During peri-implantation development, their epigenetic fates diverge with respect to XCI. Whereas extraembryonic tissues, including the primitive endoderm (PE) and the trophectoderm (TE), maintain imprinted X^P inactivation, the epiblast lineage undergoes XCR and initiates a new round of inactivation—this time randomly without a parent-of-origin bias (Mak et al., 2004).

Mechanisms

Cis-*Acting Control Regions*

Both XCI and genomic imprinting are regulated by *cis*-acting master control regions. For XCI, a single *Xic* has been mapped to a 100–500 kb region (Brown et al., 1991b; Lee et al., 1999b; Chureau et al., 2002) (Figure 3A). Genetic analyses based on knockouts, gain-of-function mutations, and transgenic overexpression have shown that the *Xic* is necessary and sufficient to regulate XCI. Deleting the noncoding locus *Xist* results in loss of silencing capability in *cis* (Penny et al., 1996; Marahrens et al., 1997), and placing the *Xic* at an ectopic location results in counting, choosing, and silencing of the autosome (Lee et al., 1996; Migeon et al., 1999; Wutz et al., 2002). The *Xic* therefore drives XCI without a requirement for additional X-specific elements, such as those that might be responsible for the spread of silencing.

Similarly, genomic imprinting is regulated by *cis*-acting ICRs that influence allelic expression across long distances. Whereas the *Xic* controls 150 Mb of a chromosome, ICRs control gene clusters of 0.1–3.0 Mb. Within the clusters, the direction of transcription and distribution of maternally versus paternally imprinted genes can vary (Figure 1). However, nearly all imprinted clusters studied to date contain at least one each of maternally expressed and paternally expressed genes. ICRs are usually just a few kilobases in length with allele-specific DNA methylation and chromatin modifications, but their ICR's location relative to the genes can also vary. Most ICRs are methylated in the female germline during oocyte growth (Bartolomei and Ferguson-Smith, 2011). A few, including the ICRs for the *H19/Igf2* and *Gtl2/Dlk1* clusters, are methylated on the paternal allele prior to birth in the male germline (Bartolomei and Ferguson-Smith, 2011) (Figure 1A). Maternally methylated ICRs typically harbor the promoter for lncRNAs, examples of which include the ICRs for *Kcnq1ot1*, *Snprn*, and *Airn* (Figures 1B–1D). In contrast, paternally methylated ICRs are intergenic (Barlow, 2011). In the case of the *H19/Igf2* locus, the ICR serves as a methylation-sensitive insulator (Figure 1A). In all cases, ICR deletions result in the loss of imprinting of multiple genes within the cluster.

Long Noncoding RNAs

The X-Inactivation Center. Noted early in the study of both phenomena, a prominent feature of the *Xic* and ICRs is their association with lncRNAs, the prototypes of which were discovered within these regions (Brannan et al., 1990; Borsani et al., 1991; Brown et al., 1991a; Lee et al., 1999a; Koerner et al., 2009). With respect to function and mechanism, the *Xic* harbors some of the best-characterized lncRNAs. The "X-inactive-specific transcript" (XIST/Xist) (Brockdorff et al., 1992; Brown et al., 1992) produces a 17–20 kb RNA that decorates

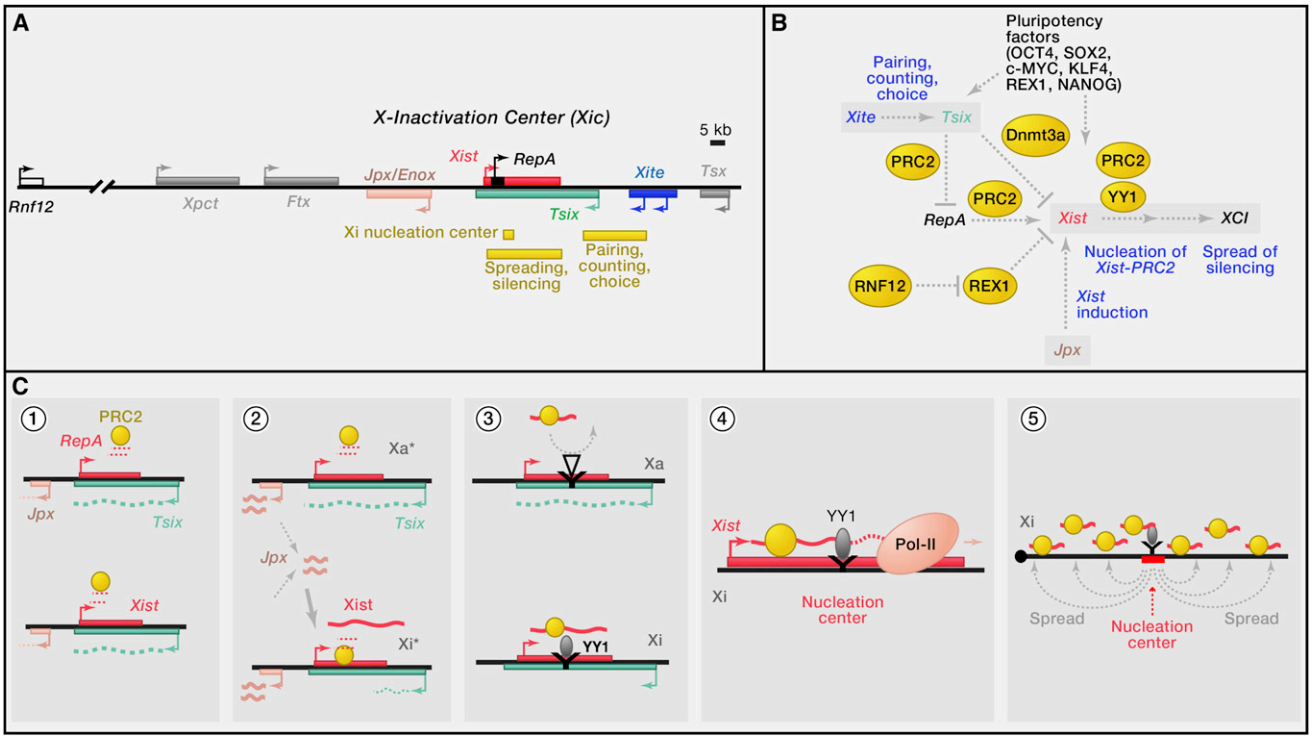

Figure 3. The Control Center and Steps of Initiation during X Chromosome Inactivation

(A) The X-inactivation center consists of multiple genes encoding lncRNA, including *Xist*, *RepA*, *Tsix*, *Xite*, *Jpx/Enox*, *Ftx*, and *Tsx*. Regions involved in various steps of XCI (counting, choice, pairing, and silencing) are delineated.

(B) Converging pathways of RNA-protein interactions during XCI. Yellow ovals represent chromatin complexes (PRC2, YY1, DNMT3a, RNF12, and REX1) that interact with indicated lncRNA or associated loci. Positive regulation shown by pointed arrows; negative regulation shown by blunted arrows. Various steps of XCI are shown in blue lettering.

(C) Initiation of XCI by lncRNA. (1) Biallelic *Tsix* prevents loading of RepA-PRC2 and initiation of XCI; (2) Two events enable *Xist* expression during cell differentiation: induction of the *Jpx* activation and monoallelic loss of *Tsix* on Xi, which allows RepA-PRC2 to load; (3) *Xist* cotranscriptionally recruits PRC2. YY1 binds Xi nucleation center, but is blocked from binding Xa; (4) *Xist*-PRC2 complex cotranscriptionally loads onto the YY1-based nucleation center; (5) From the nucleation center, *Xist*-PRC2 spreads in a *cis*-limited fashion to ~150 strong Polycomb stations, which in turn spread H3K27me3 via 3,000–4,000 moderate Polycomb sites.

the X chromosome during the initiation of XCI (Clemson et al., 1996). *Xist* is expressed only from the Xi and is required for whole-chromosome silencing (Penny et al., 1996; Marahrens et al., 1997). Xist RNA directs chromatin and transcriptional change by binding Polycomb repressive complex 2 (PRC2), the epigenetic complex responsible for trimethylation of histone H3 at lysine 27 (H3K27me3), and targeting PRC2 to the Xi (Zhao et al., 2008) (Figure 3B). This discovery suggests RNA as a crucial guiding factor in Polycomb targeting. However, PRC2 targeting and binding to the chromatin are biologically separable, as indeed chromatin loading is precluded when Xist's antisense partner, Tsix (Lee et al., 1999a), is expressed in *cis* (Zhao et al., 2008). Only when Tsix expression is downregulated during development does the Xist-PRC2 complex load onto the Xi "nucleation center" within *Xist*'s exon 1 (Jeon and Lee, 2011). The nucleation center consists of three binding sites for the transcription factor, YY1, a protein bound only to the Xi allele. By cotranscriptionally tethering Xist RNA to the *Xic*, YY1 bridges PRC2, Xist RNA, and Xi chromatin (Figure 3B).

From the nucleation center, PRC2 spreads initially to ~150 strong binding sites along the future Xi, concentrating predominantly within bivalent domains coinciding with CpG islands (Pinter et al., 2012) (Figure 3C). As XCI proceeds, the coating of the future Xi by Xist RNA correlates with recruitment of 3,000–4,000 moderate Polycomb sites, most of which are intergenic, nonbivalent, and lack CpG islands. The moderate sites cluster around strong sites and facilitate the spreading of H3K27me3 in a graded concentration relative to strong sites. Interestingly, Polycomb stations are not enriched for the LINE1 repeats previously hypothesized to influence spreading (Lyon, 2003; Chow et al., 2010). Thus, the spreading of XCI is also controlled by Xist RNA and is governed by a hierarchy of defined Polycomb stations along the Xi.

Xist is itself controlled by other lncRNAs, some acting negatively (e.g., Tsix), others positively (e.g., Jpx). Tsix RNA, the antisense partner of Xist RNA (Lee et al., 1999a), represses *Xist* in several ways. First, *Tsix* coordinates X chromosome pairing to generate the epigenetic asymmetry required for selection of future Xa and Xi (Bacher et al., 2006; Xu et al., 2006; Xu et al., 2007). Second, Tsix also recruits DNA methyltransferase (Dnmt3a) to silence *Xist* (Sado et al., 2005; Sun et al., 2006). Third, it blocks recruitment of PRC2 to Xist

(and RepA, see below), potentially by binding PRC2 and titrating it from Xist/RepA RNAs (Zhao et al., 2008). Tsix also duplexes with Xist/RepA RNA (Ogawa et al., 2008) and possibly serves as decoy for PRC2 recruitment (by titrating Xist-RepA RNA or PRC2). In these ways, Tsix determines allelic choice by repressing *Xist* transcription on one allele (Figure 3B).

Xist is regulated positively by Jpx RNA (Tian et al., 2010) (Figure 3C). Deleting *Jpx* abolishes *Xist* activation, indicating that *Jpx* is a positive regulator. Because knocking down the RNA recapitulates the deletion, Jpx must function as an RNA and not merely through its act of being transcribed. Moreover, because Jpx expression from an autosomal transgene can rescue the X-linked deletion, Jpx RNA is *trans* acting, unlike other elements of the *Xic*. The 1.6 kb RepA RNA (intragenic to Xist) has also been implicated as a potential activator of *Xist* expression, as its expression appears to be necessary for *Xist* upregulation (Zhao et al., 2008) and deleting the Repeat A motif (Hoki et al., 2009) abolishes *Xist* induction. The linked noncoding *Ftx* locus has also been suggested to regulate *Xist*, as deleting *Ftx* in male cells has mild effects on the chromatin profile of *Xist* (Chureau et al., 2011), but its effects in female cells are currently unknown. These *Xist* regulators work in parallel with the E3 ubiquitin ligase, RNF12, encoded by an X-linked gene near the *Xic* (Figures 3A and 3B): Its overexpression causes partial derepression of *Xist* (Jonkers et al., 2009), and knockouts of *Rnf12* block imprinted XCI and delay random XCI (Shin et al., 2010; Barakat et al., 2011). The pluripotency factor, REX1, has been identified as a target of RNF12 (Gontan et al., 2012). It is thought that elimination of REX1 binding to the *Xist* promoter facilitates activation of *Xist*. These studies collectively point to central functions for lncRNA-protein interactions, with the lncRNAs targeting epigenetic complexes, serving as antisense inhibitors, and activating sense transcription.

Imprinting Clusters. Every imprinted cluster harbors lncRNAs (Figure 1), many of which originate within or near ICRs. These lncRNAs are themselves imprinted. The most common mechanism used for imprinting relies on expression of a lncRNA in *cis* and exploits much of what has been identified for silencing of the X chromosome during XCI (Figure 1). There are currently six well-characterized clusters of imprinted genes (along with at least nine additional less well-studied clusters), including *Igf2r/Airn*, *Kcnq1*, *Snprn/Ube3A*, *Gnas*, *Igf2/H19*, *Dlk1/Gtl2* (Barlow, 2011). All of these clusters contain lncRNAs. Three imprinted lncRNAs are long mature RNAs: *Airn* is 108 kb (Lyle et al., 2000), *Kcnq1ot1* is approximately 100 kb (Pauler et al., 2012), and, *Ube3a-ATS* may be in excess of 1,000 kb (Pauler et al., 2012). The *Gtl2* lncRNA contains multiple alternatively spliced transcripts, however, downstream intergenic transcription has also been noted, suggesting longer RNAs are likely (Tierling et al., 2006). *Nespas* lncRNA exceeds 27 kb (Robson et al., 2012).

Experiments that directly test the role of the lncRNA itself have now been performed for a number of loci (*Airn*, *Nespas*, *Kcnq1ot1*, and *Ube3aats)*. Thus far, all have been analyzed by genetic manipulation of the endogenous locus to truncate the lncRNA by insertion of a polyadenylation signal. The 108 kb *Airn* lncRNA has been examined in the most detail. Initially, Barlow and colleagues reported that truncation of Airn to 3 kb in a mouse model suggested that the lncRNA itself is necessary to silence all 3 mRNA genes in the *Igf2r* cluster, indicating a clear regulatory role for this lncRNA (Sleutels and Barlow, 2002) (Figure 1A). Similarly, truncation of the ~100 kb *Kcnq1ot1* lncRNA to 1.5 kb showed that this lncRNA was directly needed to silence all 10 mRNA genes in the larger *Kcnq1* cluster (Mancini-Dinardo et al., 2006) (Figure 1B), and truncation of the ~27 kb *Nespas* lncRNA showed it was necessary to silence the overlapped *Nesp* gene in the *Gnas* imprinted cluster (Williamson et al., 2011). Lastly, truncated *Ube3a-ATS* in an embryonic stem (ES) cell model resulted in activation of paternal *Ube3a* (Figure 1D), consistent with the role for the *Ube3a-ATS* lncRNA in repressing paternal *Ube3a* in neurons (Meng et al., 2012) (Figure 1).

At this point, it is not clear how the lncRNAs silence imprinted genes in *cis*. One possibility is that they overlap adjacent imprinted genes and the sense-antisense overlap causes a form of transcriptional interference of a promoter or an enhancer, which in turn affects transcription from the mRNA promoter (Pauler et al., 2012). In this case, the first event could be silencing of the overlapped promoter or enhancer followed by accumulation of repressive chromatin that can spread and induce transcriptional gene silencing throughout the cluster. Evidence for this model was recently obtained by Latos and colleagues by generating a series of recombinant endogenous chromosomes at the *Igf2r/Airn* locus in ES cells (Latos et al., 2012) (Figure 1C). Analogous to XCI, the onset of allele-specific expression at this locus in the embryo can be recapitulated by ES cell differentiation, where *Igf2r* is initially biallelically expressed but the initiation of *Airn* expression results in *Igf2r* imprinting (Latos et al., 2009). To test whether *Airn* transcription or the lncRNA itself was required for *Igf2*r silencing, *Airn* was shortened to different lengths and it was shown that silencing only required *Airn* transcription overlap of the *Igf2r* promoter, which interferes with RNA polymerase II recruitment (Latos et al., 2012). This model suggests that *Airn* acts predominantly through its transcription, rather than as a lncRNA like Xist.

It is, however, also possible that imprinted lncRNAs act by coating the local chromosomal region and directly recruiting repressive chromatin proteins to the imprinted cluster, in a manner similar to *Xist* lncRNA. Many imprinted lncRNAs, such as Gtl2 and Nespas, appear to form a complex with Polycomb proteins (Pandey et al., 2008; Zhao et al., 2010). Evidence for a function of the lncRNA in recruitment of histone posttranslational modification machinery comes from experiments in placental tissues. RNA fluorescence in situ hybridization experiments showed that *Airn* and *Kcnq1ot1* form RNA clouds at their site of transcription and are associated with a repressive histone compartment and Polycomb proteins (Nagano et al., 2008; Pandey et al., 2008; Terranova et al., 2008; Redrup et al., 2009). This nuclear compartment is also devoid of RNA polymerase II and exists in a three-dimensionally contracted state. Other studies on the *Airn* lncRNA go further in suggesting that the lncRNAs actively recruit repressive histone modifications (Nagano et al., 2008) but only in the placenta. In this latter case, *Airn* was shown to actively recruit the histone H3 lysine 9 methyltransferase, G9a, and paternal-specific silencing of the *Slc22a3* gene but not the *Igf2r* gene, was dependent on

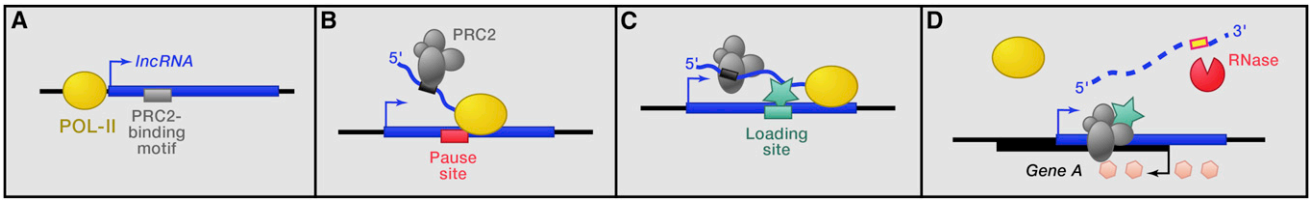

Figure 4. LncRNAs Tether Epigenetic Complexes to Chromatin, Enabling Allelic, and Locus-Specific Regulation
(A–C) LncRNA transcribed by RNA polymerase II (POL-II) (A) cotranscriptionally binds to an epigenetic complex (such as PRC2) (B), which loads onto chromatin through DNA-bound factors such as YY1 (for Xist RNA) (C).
(D) Epigenetic modifications silence the linked gene. Rapid lncRNA turnover prevents diffusion and action at ectopic loci. Adapted from Lee, 2012.

G9a—in a mechanism that contrasts with the promoter-transcription model hypothesized on the basis of transcript truncation experiments in somatic lineages (Latos et al., 2012). These experiments indicate that lncRNA mediated silencing of imprinted genes may depend on different downstream mechanisms.

A new class of lncRNAs was recently discovered, sno-lncRNAs, that arise from the imprinted Prader-Willi Syndrome (PWS) critical region of human chromosome 15 (Yin et al., 2012). Intriguingly, these lncRNAs, which have a snoRNA sequence at each end as well as intervening sequence, accumulate near the sites of synthesis and strongly associate with Fox family splicing regulators and alter splicing. The investigators hypothesize that the sno-lncRNAs in the PWS locus function as molecular sinks for Fox2, acting locally to alter splicing patterns in specific subnuclear neighborhoods. Thus, the mechanisms by which lncRNAs operate at imprinted loci are diverse.

Why Are lncRNAs Central to Imprinting and XCI?. It has been argued that lncRNAs make ideal factors for allelic regulation (Lee, 2012). Indeed, lncRNA's tethering capabilities and potential for fast turnover renders them excellent allelic markers. These transcripts are tethered to the site of synthesis through the RNA polymerase II transcription complex and can therefore function as allele-specific tags (Figure 4). As shown by Xist and RepA RNA, such long transcripts can cotranscriptionally capture chromatin complexes while tethered to the site of transcription (Zhao et al., 2008). Tethering could be aided by bridge proteins, such as YY1 in the case of Xist RNA (Jeon and Lee, 2011). Rapid RNA turnover after transcription would prevent diffusion to ectopic sites. At the *Xic*, the decoying effect of Tsix for Polycomb proteins would be limited to the *Xic* by Tsix's very short half-life (30–60 min, the time required to synthesize the 40 kb RNA; Sun et al., 2006) so that effective concentrations would only be reached at the site of synthesis. Whereas lncRNAs can mark alleles, proteins cannot retain such allelic memory because their transcriptional origin is lost when mRNA is shuttled to the cytoplasm.

Another property of lncRNAs is their ability to specify a unique address (Lee, 2012). Although transcription factors can also recruit epigenetic complexes, lncRNAs offer the possibility of targeting to a single location. Transcription factors typically target complexes to multiple genomic locations because they recognize short DNA motifs that occur hundreds to thousands of times in the genome. In contrast, lncRNAs such as Tsix and RepA/Xist occur only once in the genome. Because of this uniqueness, lncRNAs can deliver epigenetic complexes to a single address, offering a regulatory specificity not possible with proteins or small RNAs. These properties may explain why the protein-coding region syntenic to the present-day *Xic* was rapidly transformed into a noncoding landscape ~150 million years ago when random XCI first appeared in eutherian mammals (Duret et al., 2006). Prior to this time, *Xist* was a ubiquitin ligase, *Lnx3*, and Jpx was a peptidase, *UspL1*. It is likely that lncRNAs evolved within imprinted domains and other locations in the mammalian genome for similar reasons. For a discussion of genome-wide lncRNAs with epigenetic functions, we refer readers to the accompanying Review by Batista and Chang on page 1298 of this issue (Batista and Chang, 2013).

Insulators

Despite the common occurrence of lncRNAs at imprinted loci, insulators may play an equally important role in imprinted regions. The insulator model, which has been described at the *Igf2/H19* locus (Figure 1A), is an evolutionarily older mechanism, components of which are conserved in marsupials (Smits et al., 2008). Key to this mechanism is CTCF-binding sites in the ICR, which exhibit insulator or enhancer blocking properties (Bell and Felsenfeld, 2000; Hark et al., 2000). On the maternal allele, CTCF binds to the ICR and blocks the access of *Igf2* to enhancers shared with *H19*, which are located downstream, thereby allowing *H19* exclusive enhancer access. On the paternal allele the ICR acquires DNA methylation in the male germline, preventing CTCF binding, allowing *Igf2* interaction with the enhancers and paternal-specific expression (Figure 1A). The presence of DNA methylation on the paternal ICR leads to secondary methylation of the *H19* promoter and paternal-specific *H19* silencing (Thorvaldsen et al., 1998). The involvement of CTCF in the insulator model has prompted the identification of CTCF-binding sites at other imprinted genes such as *Rasgrf1*, *Grb10*, and *Kcnq1ot1*, indicating that the insulator model may operate in other imprinted clusters. CTCF sites have also been identified within the *Xic* in regions important for imprinted XCI (Chao et al., 2002); however, it is currently unknown if CTCF is central to imprinting the X. Insulator-based and lncRNA-based models are not mutually exclusive.

The Enigma of Imprinted XCI

Imprinted XCI in the Mouse

A further consideration of imprinted XCI is worthwhile for its mechanistic differences and implications for human development. The mechanism of X-imprinting not only differs from

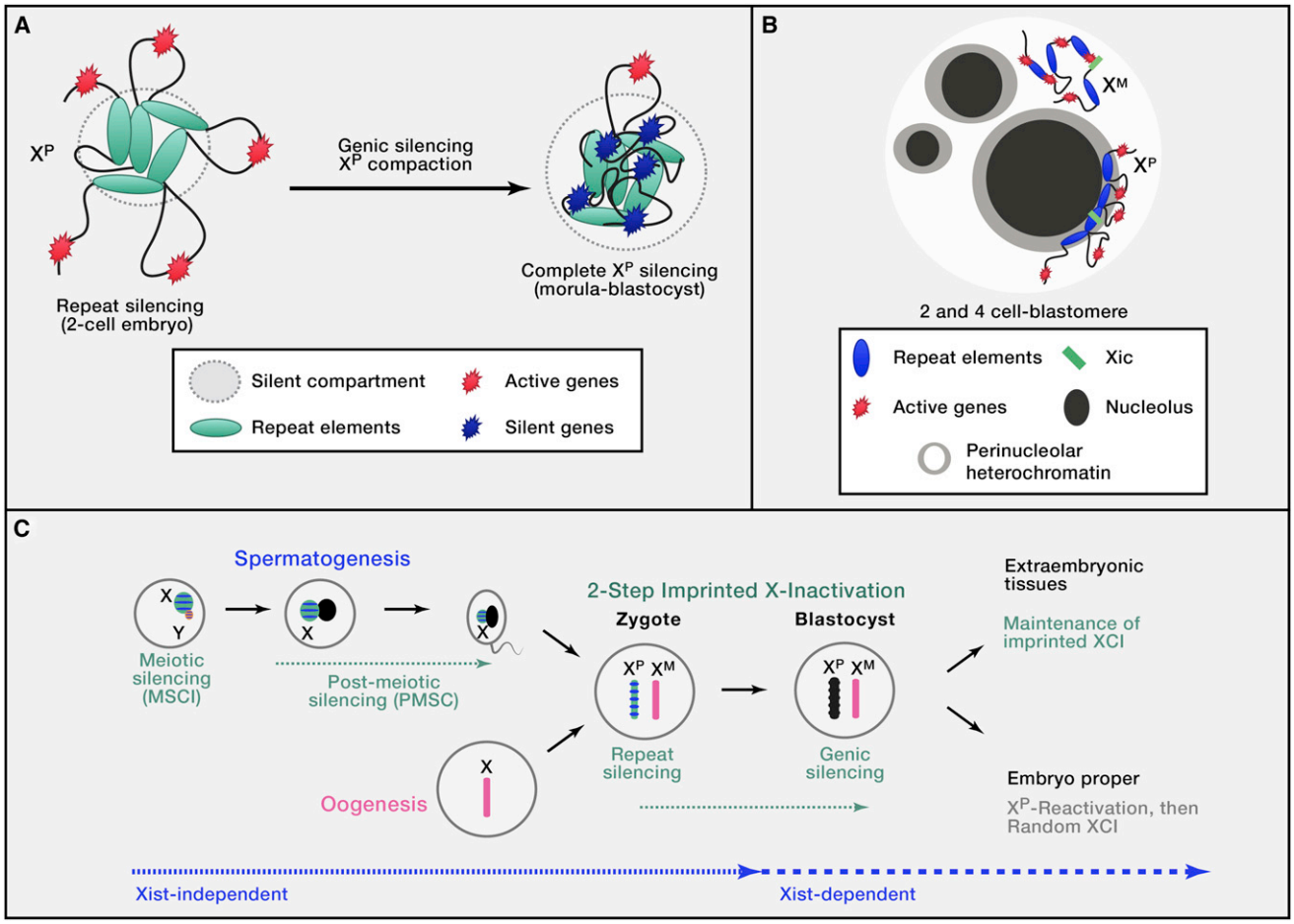

Figure 5. Imprinted XCI in the Mouse

(A) Pictorial representation of genic localization into the preformed silent compartment during imprinted XCI. X^P repeats form a silent compartment next to the nucleolus by the two-cell stage and, although Xist RNA localizes within it, formation of this silent compartment does not require *Xist*. The repeats could potentially contribute to imprinted XCI by setting up a silencing compartment next to the nucleolus. The silent compartment is present by the two-cell stage and enlarges as genic loci are translocated into it and become silenced. Genic silencing depends on *Xist*. X^P silencing is completed by the blastocyst.

(B) Pictorial representation of X^P and X^M in the early mouse embryo. Repeat elements of X^P create the silent perinucleolar compartment, whereas X^M and active genic loci of X^P reside in repeat-expressing regions.

(C) Hypothesis: developmental history of the X chromosome from gamete to embryo. Hypothesized events in imprinted XCI of the mouse are shown. In the male germline, during the first meiotic prophase, the X and Y are inactivated by MSCI and remain suppressed through spermiogenesis as PMSC. This germline-inactivated X may be passed onto the next generation with its repeats preinactivated. In the two-cell mouse embryo, repetitive elements on X^P are already suppressed in an *Xist*-independent manner. X^P genic silencing occurs progressively during preimplantation development, strictly depends on *Xist*, and is completed in the blastocyst stage. Thus, imprinted XCI in the mouse embryo is a two-step process, with repeat silencing (*Xist*-independent) occurring prior to genic silencing (*Xist*-dependent). Repeat silencing could account partly for the transgenerational information (the imprint) involved in X^P silencing. The maternal germline also plays a crucial role in imprinted XCI by marking the future X^M.

Adapted from Namekawa et al. (2010).

random XCI but also differs between the imprinted marsupial and eutherian forms. In mouse imprinted XCI, X^P-repeat silencing precedes genic inactivation (Figure 5A) (Namekawa et al., 2010). The repeats form a silent compartment next to the nucleolus by the two-cell stage and, although Xist RNA localizes within it, formation of this silent compartment does not require *Xist*. Repeats could potentially contribute to imprinted XCI by setting up a silencing compartment next to the nucleolus (Figure 5B). If their silencing were indeed carried over from the male germline, repeats could account partly for the transgenerational information (the imprint) for X^P silencing.

X^P genic silencing follows repeat silencing (Namekawa et al., 2010) and occurs predominantly in the morula-blastocyst stages (Okamoto and Heard, 2006; Namekawa et al., 2010). Although one study suggests an *Xist*-independent process (Kalantry et al., 2009), the general consensus is that genic silencing depends on *Xist* (Marahrens et al., 1997; Namekawa et al., 2010). *Xist* must be marked by a second (presently unknown) imprint that would promote imprinted genic XCI (Figure 5C). In the mouse, *Xist* and *Tsix* are opposing regulatory factors for imprinted genic silencing, as they are for random XCI. Deleting *Xist* from X^P precludes placental XCI (Marahrens et al., 1997),

whereas deleting *Tsix* from X^M compromises maternal-specific protection from imprinted silencing in the placenta (Lee, 2000; Sado et al., 2001). Thus, the *Xic* plays at least a partial role in imprinted XCI in eutherian mammals.

Imprinted XCI in Marsupials

The eutherian *Xic* is not recognizable in the marsupial (Duret et al., 2006). The idea of an *Xist*-independent mechanism based on repeat silencing raises the possibility of a similar mechanism in marsupials. Notably, the opposum male germline demonstrates postmeiotic silencing of X-linked repeat elements (Namekawa et al., 2007), but whether silencing is carried over into the embryo is unknown. The recent identification of *RSX* indicates that a lncRNA like *XIST* may be present (Grant et al., 2012). The 27 kb RSX transcript also "coats" the marsupial Xi and is specifically expressed in female cells. Introduction of *RSX* transgenes into mouse ES cells results in partial silencing of three autosomal genes near the site of integration. These findings suggest that RSX may be the XIST equivalent in opposum, though an *RSX* knockout has not been performed and the two lncRNAs do not possess obvious homology. Like in the mouse, an *XIC* mechanism may occur alongside a repeat-silencing process to implement imprinted XCI.

Imprinted XCI in Humans?

The question of whether imprinted XCI occurs in the human placenta has not been resolved, but implications for human development are evident. In several studies, examination of single X-linked genes from a small number of placentae suggested preferential maternal expression (e.g., Harrison and Warburton, 1986). Using transdifferentiation of a female human ES line into trophoblast cells, another study found that *FMR1* was expressed only from one X, consistent with imprinting (Dhara and Benvenisty, 2004). However, other studies have detected expression from both X^M and X^P (Moreira de Mello et al., 2010; Okamoto et al., 2011; Peñaherrera et al., 2012); and, in a nonhuman primate model, XIST was detected from either X^M or X^P of the trophectoderm (Tachibana et al., 2012). The fact that the X chromosome contributing to Turner (XO) and Klinefelter (XXY) syndrome could be of either X^M or X^P origin (Skuse et al., 1997; Skuse, 2000, 2005) further argues against imprinting. Although the preponderance of evidence may be against imprinted XCI in human placentae, there is the intriguing possibility of X-imprinting in the brain as a basis for male-female differences in behavior and prevalence of autism (Skuse, 2000) (more below). The question of imprinting therefore bears significance for human development and disease, particularly where X-linked mutations may contribute to early fetal loss, and congenital or cognitive defects.

Human Diseases and Conditions

Congenital Diseases of Imprinting

Because of parental-origin effects, human disease syndromes can result from genetic or epigenetic abnormalities on only a single parental allele. In fact, most well-defined imprinted gene clusters are associated with human diseases (Thorvaldsen and Bartolomei, 2007). Interestingly, aberrant expression of ICR-associated lncRNAs may be implicated in various imprinting disorders. Two of the best-studied imprinting syndromes, PWS and Angelman (AS) syndromes, map to human chromosome 15 (Buiting, 2010). PWS involves loss of function of a number of genes on 15q11-13, including *SNORD116*. People with PWS are obese and have reduced muscle tone and mental ability. AS syndrome is a complex disorder of the nervous system that arises from loss of function of the *UBE3A* gene (Figure 1D). AS symptoms include delayed development, intellectual disability, and severe speech impairment. Most PWS and AS cases involve large deletions containing the imprinted genes from the chromosome on which they are expressed. In PWS, there is biallelic repression of the ICR-associated lncRNA; in AS, the lncRNA is biallelically expressed. A smaller number of cases arise from either deletion or aberrant allelic DNA methylation of the ICR, leading to expression changes. With the recent identification of the new class of lncRNAs, sno-lncRNAs, it is likely that absence of the sno-lncRNA in the PWS critical region impairs brain-specific splicing possibly due to mislocalization of Fox splicing factors.

Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder, and Silver-Russell syndrome (SRS), an undergrowth and asymmetry disorder, are two other well-studied imprinting disorders that map to human chromosome 11p15.5, where *IGF2* and *H19* reside (Figure 1A). Unlike PWS and AS, the majority of individuals with BWS or SRS have epigenetic errors. For example, over half of BWS cases exhibit loss of methylation at the *KCNQ1* ICR, which results in biallelic expression of the *KCNQ1OT1* lncRNA (Weksberg et al., 2005) (Figure 1B). Inappropriate expression of the lncRNA may lead to aberrant repression of associated disease genes in *cis*—in this case, *CDKN1C* was silenced. Additionally, some BWS patients exhibit overexpression of *IGF2*. Most of these cases have small deletions in the ICR on the maternal allele, which disrupts the CTCF-dependent insulator, leading to biallelic *IGF2* and loss of *H19* expression (Riccio et al., 2009). Curiously, the remaining ICR sequences in these individuals are hypermethylated. Many individuals with SRS have an opposite epigenetic phenotype where the ICR is unmethylated, resulting in biallelic *H19* expression and loss of *IGF2* expression. In many of these cases, it is unclear what event leads to DNA hypomethylation but in some cases, multiple imprinted loci exhibit loss of ICR methylation (Azzi et al., 2010). Significantly, some examples of multilocus loss of imprinting involve mutations in ZFP57, a zinc finger protein involved in the postfertilization maintenance of genomic imprints, which was first reported in individuals presenting with transient neonatal diabetes (Mackay et al., 2008). It is possible that yet-to-be identified proteins are mutated in other cases involving loss of methylation. Alternatively, early environment insults can affect DNA methylation patterns (see Imprinting and Assisted Reproductive Technology as an example).

X-Linked Influences on Disease, Cognition, and Behavior

The X chromosome is home to nearly 1,000 genes, many of which result in discernible human phenotypes when mutated. X-linked diseases result from single-gene mutations, which can be classified as dominant or recessive, with the former manifest in both XX and XY individuals and the latter manifest primarily in XY individuals because they lack a wild-type allele. X-linked mutations can cause serious disease, such as hemophilia A (*FVIII*), Duchenne muscular dystrophy (*DMD*), Rett syndrome

(*MECP2*), and fragile X syndrome (*FMR1*), or less serious conditions, such as red-green color blindness and male-pattern baldness. Because of differential inheritance of sex chromosomes and the hemizygous state of the X chromosome in the male population, more diseases have been described for the X chromosome than any other (Puck and Willard, 1998).

As X-linked genes have existed in the hemizygous state for much of the history of sex chromosomes, the X chromosome has been engaged in selection of sexually dimorphic traits for more than 300 million years since the X and Y began to diverge (Arnold et al., 2004; Skuse, 2005). Genes for sexual dimorphism, reproduction, and cognition are enriched on the X chromosome, with their genetic patency making them easy substrates for evolutionary selection. In mice, deleting the *Xic*-encoded lncRNA, Tsx, has been shown to reduce fear and enhance hippocampal short-term memory in male mice (Anguera et al., 2011). The fact that many X-linked genes are expressed in the brain, some in a sex-specific manner, may explain why mental retardation and autism are up to ten times more common in males, though the underlying mutations are not known for many such disorders (Skuse et al., 1997). Genetic patency of X-linked haplotypes has been hypothesized to increase the likelihood of manifesting extreme behavioral and cognitive phenotypes in males, and the likelihood would also be increased in females when the XCI pattern is skewed to favor X^M expression. XCI profiles and mosaicism vary extensively between human females, perhaps accounting for greater phenotypic variation among females (Carrel and Willard, 2005). Genes that variably escape XCI also contribute to this effect (Berletch et al., 2011). In the mouse, X-linked modifiers such as the *Xce* can skew XCI ratios (Cattanach and Isaacson, 1967; Percec et al., 2002; Thorvaldsen et al., 2012), providing a mechanism by which nonrandom XCI patterns could be generated. Nonrandom XCI is also not uncommon in human females (Puck and Willard, 1998).

In the area of cognitive and behavioral development, the study of X chromosome monosomies (XO, Turner syndrome) has played a major role in elucidating X-linked contributions. Turner syndrome girls usually have normal verbal intelligence but are less developed in spatial and mathematical skills. By comparing Turner syndrome girls who inherited their X chromosome from mother (X^MO) versus father (X^PO), one study concluded that the X^P was associated with enhanced social cognitive function (Skuse et al., 1997). Despite their genotypic similarity, the epigenetically different X^PO and X^MO girls demonstrated measurable phenotypic differences in social adjustment. The fact that the X^P chromosome is normally only inherited by daughters has led some to suggest that it accounts for better social skills in girls on average. X^PO and X^MO girls also exhibit differences in visual memory and brain structure (Bishop et al., 2000; Kesler et al., 2004). Candidate genes include *USP9X*, *MAOA*, and *MAOB* (monoamine oxidases) on the short arm of the human X chromosome (Good et al., 2003; Oreland et al., 2004).

Genes on the X chromosome may be imprinted tissue specifically, particularly in the brain where many X-linked genes are expressed. A transcriptome analysis of the mouse brain suggested that hundreds of alleles on X^M may be preferentially expressed in glutamatergic neurons of the female cortex (Gregg et al., 2010). Although X^P alleles are not silenced, they are expressed at lower levels. This type of partial imprinting could contribute to cognitive and behavioral differences. Follow-up analyses have argued that the allelic skewing called by whole-transcriptome analyses may have been an aberration caused by unappreciated statistical limitations of a novel technology (DeVeale et al., 2012; reviewed in Kelsey and Bartolomei, 2012). Thus, the question of how many and in what tissues imprinted X-linked genes may occur in eutherian mammals remains open. This clinically important area has been underexplored.

Xist, the X Chromosome, and Cancer

An association between the X chromosome and cancer has been noted since the discovery of the Barr body (Moore and Barr, 1955; Liao et al., 2003; Pageau et al., 2007). Breast and ovarian cancer cells, for example, frequently duplicate their Xa. The correlation also holds for men, as XXY men have a 20- to 50-fold increased risk of breast cancer in a *BRCA1* background (Fentiman et al., 2006), and testicular germ cell tumors often acquire supernumerary Xs (Kawakami et al., 2003). One recent study directly tested the connection of the X to cancer by conditionally deleting Xist RNA in the blood lineages of mice (Yildirim et al., 2013). This deletion resulted in overexpression of the X chromosome and a fulminant hematologic cancer known as "mixed MPN/MDS" (myeloproliferative neoplasm, myelodysplastic syndrome), a cancer that includes chronic myelomonocytic leukemia, erythroleukemia, histiocytic sarcoma, and bone marrow fibrosis. The cancer is female specific and 100% penetrant. Intriguingly, in humans, MDS is more common in women, with noted *XIST* deletions and X chromosome duplications occurring in MPN, MDS, and myeloid cancers (see references within Yildirim et al., 2013). The association is not restricted to women, as extra X chromosomes are seen in a range of leukemias in both sexes. The mouse study showed that loss of *Xist* perturbed maturation as well as longevity of hematopoietic stem cells. Thus, *Xist* plays a role not only in dosage compensation but also in suppressing cancer and preserving function of adult stem cell populations. This study illustrates the importance of studying lncRNA function not only in cells ex vivo but also within the context of the organism in vivo.

Epigenetic Reprogramming in Human Stem Cells

Xist RNA also influences the pluripotent stem cell population, as shown by recent studies of induced pluripotent stem cells (iPSC) in regenerative medicine. In mice, XCI is tightly linked to cell differentiation in the epiblast and the possession of two Xa is a hallmark of pluripotent cells of both mouse ESC and iPSC (reviewed in Minkovsky et al., 2012). The tight linkage is explained by the physical convergence of many pluripotency factors, such as OCT4, SOX2, NANOG, and REX1, at the *Xic*, specifically within control regions of *Xite*, *Tsix*, and *Xist* (Navarro et al., 2008; Donohoe et al., 2009; Navarro et al., 2010) (Figure 3B). Binding of pluripotency factors to these regions blocks initiation of XCI, and the loss of binding during cell differentiation creates a permissive state for the initiation of XCI.

XIST currently provides one of a few tangible readouts for stem cell quality. In human ESC (hESC) and iPSC (hiPSC), XIST expression and XCI do not necessarily occur in the expected manner. Female hESC and hiPSC lines occur in three different epigenetic groups based on XIST expression

(Silva et al., 2008) (Minkovsky et al., 2012). "Class I" cells are most similar to mESC in that they have two Xa in the undifferentiated state. When placed in differentiation conditions, Class I cells express XIST and initiate XCI. In contrast, "Class II" cells already express XIST and carry one Xi, even before growth under differentiation conditions. Finally, "Class III" cells once expressed XIST but irreversibly lost its expression, with evidence of partial X reactivation (Shen et al., 2008; Silva et al., 2008; Anguera et al., 2012; Tomoda et al., 2012). Epigenetic fluidity is evident through irreversible progression from Class I to II to III states (Tchieu et al., 2010; Anguera et al., 2012; Mekhoubad et al., 2012). Class I is transient, whereas Class III is dominant and stable.

Although failure of XIST expression is lethal in vivo (Penny et al., 1996; Marahrens et al., 1997), loss of XIST does not have the same dire consequences ex vivo, though these cells lack full developmental potential (Silva et al., 2008; Anguera et al., 2012). Class III hiPSCs have limited differentiation capability (Anguera et al., 2012; Mekhoubad et al., 2012), In a xenograft model, Class III hiPSCs produce cystic teratomas composed of simple cystic epithelia and undifferentiated mesenchyme, whereas Class II cells produce well-differentiated structures of three germ layers. Given the tumorigenic phenotype of the murine *Xist* deletion (Yildirim et al., 2013), most concerning would be the potential of XIST-negative hIPSC lines to cause cancer when introduced in vivo in the clinical setting. Indeed, Class III hiPSC also showed partial X reactivation, faster doubling times, and a distinct gene expression signature of cancer cells (Anguera et al., 2012), urging further careful consideration before using hiPSCs in regenerative medicine.

Genomic imprinting also contributes to quality of human and mouse iPSC (Pick et al., 2009; Sun et al., 2012). The imprinted state of the imprinted *Dlk1-Dio3* locus—in particular the expression of Gtl2 (aka Meg3) lncRNA—has been at the center of attention. One study found that mouse iPSC clones with aberrant *Dlk1-Dio3* imprinting and low Gtl2 expression contributed poorly to chimeras (Stadtfeld et al., 2010), whereas another did not observe a difference (Carey et al., 2011). There is, however, general agreement that loss of imprinting at this locus resulted in lower efficiency of generating entirely iPSC-derived mice (Stadtfeld et al., 2010; Carey et al., 2011; Stadtfeld et al., 2012). With further investigation, it is likely that other imprinted loci will affect stem cell quality. Nonetheless, despite a number of claims that imprinting is aberrant, iPSCs can be an important tool for studying imprinting perturbations in inaccessible cell types such as neurons in AS (Chamberlain et al., 2010).

Imprinting and Assisted Reproductive Technology

Related to the issue of epigenetic change within imprinted and X-linked loci in stem cells ex vivo is the question of whether in vitro culture of early human embryos during use of ART might have similar effects on imprinting and XCI. The ex vivo manipulations utilized during ART coincide with the developmental stages in which genome-wide epigenetic reprogramming occurs (i.e., oocyte growth and preimplantation development). The use of ART procedures to help couples with fertility issues conceive children of their own has doubled in the last decade. In 2009, ART contributed to 1.4% of all U.S. births (Sunderam et al., 2012). Nevertheless, there is growing concern about the safety of these procedures (Manipalviratn et al., 2009). Of particular concern, children conceived by ART have an increased incidence of rare epigenetic disorders, with most of these patients exhibiting loss of DNA methylation at ICRs (Manipalviratn et al., 2009). Specifically, cases of AS and BWS in children conceived by ART are associated with loss of methylation of the *SNPRN* and *KCNQ1* ICRs, respectively, which result in biallelic expression of the lncRNAs and loss of expression of *UBE3A* and *CDKN1C*, respectively (Figure 1). Consistently, animal studies have demonstrated that embryo culture and embryo transfer as well as hormonal treatments, which are integral components of ART, disrupt normal epigenetic programming in embryonic and extraembryonic lineages (Mann et al., 2004; Fortier et al., 2008; Rivera et al., 2008), although the mechanism for this disruption remains poorly understood. Thus, a greater understanding of in vitro effects on epigenetic regulation during ART is a rising need from a public health perspective of industrialized countries.

Conclusions and Therapeutic Prospects

XCI, genomic imprinting, and lncRNA clearly have major implications for public health. Yet, in the arena of preventive, diagnostic, and therapeutic medicine, few strategies have targeted regulatory factors for imprinted genes and the *Xic* to control X-linked disease and conditions. This holds true also for regenerative medicine and stem cell biology, where ex vivo cellular manipulations have not universally considered the impact of imprinting and XCI, in spite of converging indications that these processes impact production, maintenance, and overall quality of stem cells.

On a hopeful note, proof-of-concept was reported in one recent study. One of the most intriguing aspects of disorders that involve monoallelically expressed genes is the prospect for therapy that involves derepressing the silenced allele in situations where the expressed allele of an imprinted gene is deleted or contains a loss of function mutation. A recent success was reported for AS, where a screen revealed that small molecule topoisomerase inhibitors reactivated the silenced *UBE3A* gene and repressed the ICR-associated antisense RNA (Huang et al., 2012). Ironically, however, because of the clustering of imprinted genes, the biallelic activation of *UBE3A* was accompanied by the loss of expression of the paternally-expressed genes in the locus (Figure 1D). Although the mechanism for reactivation is unclear, these strategies offer hope and suggest that other loci could be subject to similar screens.

The successful reactivation of the silent copy of *UBE3A* raises hopes that a treatment for various X-linked diseases might be similarly achieved. Of particular interest has been Rett syndrome, a neurologic disorder caused by mutations in *MECP2*. The syndrome affects girls and is manifested by a reversal of developmental milestones after the first year of life (the disease is fatal in newborn males). Because Rett syndrome is not accompanied by neurodegeneration, efforts have been devoted to restoring expression of MECP2 after birth in hopes of reversing the symptoms. Intriguingly, mouse models have shown that restoration of MECP2 expression after disease first becomes symptomatic can reverse the neurologic defects

(Giacometti et al., 2007; Guy et al., 2007). Because the possibility that restoration of MECP2 expression might similarly cure Rett syndrome in humans, ongoing studies are now aimed at reactivating the wild-type copy of *MECP2* in affected girls through small molecules that target chromatin modifiers and other regulators of XCI and XCR.

Furthermore, with knowledge that loss of XIST expression and/or overdosage of the X chromosome could result in blood cancer (Yildirim et al., 2013), cancer therapeutics might similarly be directed at genes on the X chromosome. In the future, in addition to *trans*-acting factors such as topoisomerases, therapeutic strategies could be targeted at control regions (ICRs, *Xic*) or lncRNAs that regulate crucial genes in *cis*. For example, it may be productive to determine ways to control expression of XIST RNA, GTL2, and other imprinted genes. We may be some ways from realizing commercial products, but the technologies to develop them are evolving rapidly and may soon enable us to produce drugs to influence cellular reprogramming ex vivo and to treat human diseases and conditions in vivo.

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